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간세포암 유전체의 메틸화 분석: 탐색적 연구

Explorative analysis for DNA methylation in hepatocellular carcinoma

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서울대학교 대학원

중개의학과 분자종양의학 전공

박진현

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Abstract

Explorative analysis for DNA methylation in hepatocellular carcinoma

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Introduction: Hepatocellular carcinoma (HCC) is one of the most common cancers and epigenetics have been recognized to play a key role in its pathogenesis. This research aimed to evaluate the predictive value of DNA methylation profiles for late recurrence in HCC after resection.

Methods: A total of 184 patients who underwent curative resection at a single institute from 2011 to 2016 were prospectively enrolled. Illumina Infinium HumanMethylation EPIC 850K BeadChip (Illumina, CA, USA) arrays were used to examine DNA methylation profiles in HCC tumors and adjacent nontumorous liver tissues.

Results: Among the initial 184 patients, we excluded two, for whom tumor tissue was inadequate to perform tumor DNA extraction, in addition to 42 patients who presented with disease recurrence within 1

year after surgery. Of the remaining 140 patients, two tumor subgroups (methylation group 1 and 2) were identified based on methylation profiles using consensus clustering. Interestingly, methylation group 1 (N = 81, 57.8%) and 2 (N = 59, 42.2%) were different from each other, and the methylation profile of group 2 was most distinct from nontumorous liver tissues. In contrast, group 1 had similar methylation profiles to nontumorous liver tissues. At the time of analysis, 28 (23.5%) patients had experienced recurrence. In methylation group 1 and 2, this was observed in 12 (14.8%) and 16 (27.1%) patients, respectively. Moreover, the median relapse-free survival (RFS) of methylation group 1 was longer than that of methylation group 2 (not reached vs 1505 days, p = 0.036). Based on univariate analysis, patients with preoperative thrombocytopenia (platelet$<100 \times 10^9$/L) had worse RFS than patients without thrombocytopenia (921 days vs not reached, p = 0.045). However, by multivariate analysis, the methylation profile was the only significant predictor of late recurrence.

Conclusions: The major finding of the present study is that late recurrence in patients who received curative resection for HCC can be predicted based on DNA methylation. Methylation group 2 was found to be associated with poorer RFS. Our data could be used to provide more personalized therapy for patients at higher risk of late recurrence.

Keywords: Hepatocellular carcinoma; DNA methylation; Epigenetics; Recurrence; Predictive marker; Prognosis
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LIST OF ABBREVIATIONS

HCC; Hepatocellular carcinoma

RFS; Relapse-free survival

HBV; Hepatitis B virus

HCV; Hepatitis C virus

PIVKA-II; Prothrombin induced by vitamin K absence or antagonist-II

AFP; Alpha-fetoprotein

ALT; Alanine aminotransferase

AST; Aspartate aminotransferase

PT; Prothrombin time

CGI; CpG islands

Introduction

Hepatocellular carcinoma (HCC) accounts for ~80% of liver cancers and is the sixth most common neoplasm and the third leading cause of cancer-related mortality in the world(1). The global incidence of HCC has been increasing, with Asian countries comprising almost 80% of patients worldwide (2, 3). In the US, the incidence of HCC has more than doubled over the last decades(4). HCC typically occurs with chronic inflammation secondary to hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, or alcoholism. A variety of minor etiologies also include primary biliary cirrhosis and hemochromatosis (5, 6). Prolonged liver damage related to these conditions is associated with the repeated regression and regeneration of hepatocytes, causing a multistep process that includes inflammation, fibrosis, cirrhosis, and finally carcinogenesis; accordingly, upwards of 90% of all HCCs arise within cirrhotic livers(7). Because of this frequent underlying cause, the treatment of patients with these liver diseases remains difficult.

Hepatic resection, liver transplantation, and tumor chemoembolization are curative treatment options for patients with early HCC (8, 9). Theoretically, liver transplantation is the gold standard for cirrhotic HCC patients within the Milan criteria because it can remove all intrahepatic cancer foci together with the underlying oncogenic

liver(10). However, because donor organs are scarce, the application of liver transplantation is limited. Thus, primary hepatic resection remains the predominant treatment option for patients with early HCC or serves as a bridge for salvage liver transplantation(11). The Milan criteria, introduced by Marraferro in 1996, restricts HCC in adults as follows: (1) single tumor diameter less than 5 cm; (2) not more than three foci of the tumor, with each one not exceeding 3 cm; (3) no major vessel invasion; (4) no extrahepatic involvement(12). The resection of HCC is considered a safe operation with low operative mortality as a consequence of advanced surgical techniques and perioperative management(13). Surgical resection is thus generally considered one of the most effective therapies for HCC patients; however, there is a high risk of recurrence with postoperative HCC. Accordingly, the postoperative outcome for this disease remains unsatisfactory, with the 3-year recurrence rate after hepatic resection being more than 50%; as such, recurrence is also the main cause of death after treatment(14). The prognosis for advanced HCC is well known to be extremely poor. One available systemic treatment option is sorafenib, which showed minimal survival benefits in a phase III clinical trial(15). It is thus obvious that the effective treatment of recurrence is important to improve outcomes after HCC resection, and accordingly, predicting recurrence has become a subject of interest. However, few systemic treatment options emphasize the need understand disease progression based on etiologic background to

improve prognostic approaches.

In HCC, alpha-fetoprotein (AFP) has been known to have diagnostic value since the 1970s, when most patients were diagnosed at an advanced stage. Recently, advanced imaging techniques can often detect small (≤ 3 cm) tumors, and therefore, the role of AFP as a diagnostic tool tend to less significant(16). Regardless, AFP is well known as a useful predictor of prognosis(17-19), and particularly recurrence. There are also many studies on the usefulness of prothrombin induced by vitamin K absence or antagonist-II (PIVKA-II) for the diagnosis of HCC and the detection of recurrent HCC after curative resection(20, 21). Over the last two decades, many prognostic staging systems using clinicopathologic data have been introduced for HCC, such as the Japan Integrated Staging (JIS) score(22, 23), the modified JIS score(24), the Cancer of the Liver Italian Program(17, 25), the Tokyo Score(26), and the Barcelona Clinic Liver Cancer staging system(27, 28). Recent studies have also developed a prognostic model for HCC recurrence, based on messenger RNA (mRNA) genetic signatures obtained from HCC resection specimens and biopsies(29-31). However, the direct translation of these prognostic models into clinical decision making has not been reported.

Recurrence in remnants of the liver can originate from either intrahepatic metastasis from the primary tumor or multiple recurrence. Recurrence can also be classified into early (≤ 1 year) and late (> 1year) recurrence after resection. Previously, some studies suggested that early

recurrence most likely originates from subclinical metastasis of primary cancers, whereas late recurrence might comprise *de novo* primary HCC in the remaining liver or multicentric disease(32, 33). Further, different risk factors are considered related to each type of recurrence. For example, advanced tumor stage, the presence of microvascular invasion, and elevated AFP levels are suggested predictive markers for early recurrence, whereas multinodularity, hepatitis activity, and cirrhosis are reportedly associated with late recurrence(32, 34, 35). Previous studies have also shown that late recurrence is related to a better outcome than early recurrence, but the underlying mechanism is still not clear(22, 36). Thus, different strategies might be considered for the prevention and management of early and late recurrences, and as such, our study focused on late recurrence.

Epigenetic mechanisms of carcinogenesis are rapidly becoming important to understand human malignancies. DNA methylation is well known to regulate cell differentiation and participate in tumorigenesis(37). Specifically, the global loss of DNA methylation is a hallmark of cancer, and this disease is also characterized by selective promoter hypermethylation. Moreover, there is evidence that epigenetic markers could be used as prognostic and predictive biomarkers in clinical practice(38). For example, the analysis of methylation has enabled the classification of colon cancer patients to predict prognosis(39). In HCC, there are few studies concerning the methylome and epidrivers, as well

as the introduction of a methylation signature that can predict patient survival(40, 41). Thus, we believe that there is a need for an accurate model based on DNA methylation to predict the probability of HCC recurrence after curative resection. We thus aimed to develop this type of model to discriminate late recurrence risk based on DNA methylation.

Material and Methods

Study population

This study was approved by the institutional review boards of Seoul National University of Hospital. Written informed consent was obtained from every patient. From 2011 to 2016, 184 patients who underwent resection of HCC at the department of Surgery, Seoul National University of Hospital, were prospectively enrolled. All 184 patients received curative resection, defined as complete excision of the tumor with clear microscopic margins and no residual tumors, as demonstrated by a CT scan after surgery. Patients regularly visited the outpatient clinic and were monitored for recurrence based on a standard protocol including serum tumor markers, α-fetoprotein (AFP) levels, and contrast CT scans or MRI every 3 months. Recurrence was classified as early (≤ 1 year) and late (> 1year) recurrence. We excluded patients who presented with recurrence within 1 year after surgery. Demographic data and clinicopathologic characteristics were obtained from hospital charts. Patients were followed up from surgical resection until death.

Clinicopathologic variables that could be potentially associated with the risk of recurrence were collected. These included 13 host related factors (sex, age: 65 year or younger or older than 65 years, HBV status, HCV status, alcohol abuse, preoperative serum albumin: < 3.3 g/dL or

≥3 .3 g/dL, bilirubin: < 1.2 μmol/L or ≥ 1.2 μmol/L, alanine aminotransferase [ALT]: < 50 IU/L or ≥ 50 IU/L, aspartate aminotransferase [AST]: < 50 IU/L or ≥ 50 IU/L, platelet counts: < 100 × 10^9/L or ≥100 × 10^9/L, prothrombin time [PT]: < 80% or ≥ 80%, Child-Pugh Grade, and nontumor liver histology) and nine tumor-related factors (serum AFP levels, PIVKA-II levels, resection margin: < 1 cm or ≥ 1 cm, tumor size: < 5 cm or ≥ 5 cam, multinodularily, venous invasion, tumor encapsulation, histologic differentiation, Milan criteria, and MoRAL criteria). Liver histology was divided into three categories based on pathology reports, including normal, chronic hepatitis, and liver cirrhosis. We did not offer any adjuvant treatment.

Data generation

Among the 184 patients, tumor tissue was inadequate to extract DNA in two patients. Frozen HCC tissues of the remaining 182 Korean patients were prepared for this study. DNA from tissue specimens was extracted using the MagListo™ 5M Genomic DNA Extraction Kit (Bioneer) following the manufacturer's instruction. DNA concentrations were quantified using a Qubit fluorometer. Then, 600 ng of DNA from each specimen was used to generate an Illumina Infinium HumanMethylation EPIC 850K BeadChip (Illumina, CA, USA) with an Illumina iScan System (Illumina, CA, USA) using the manufacturer's standard protocol. Illumina Infinium HumanMethylation EPIC

BeadChips contain the DNA methylation values for more than 850,000 CpG sites for the resolution of every single nucleotide.

Processing DNA methylation data

We performed methylation data pre-processing using the minfi R package in R. The raw IDAT files of liver tumor tissue samples were processed and background correction and dye bias were performed. Measuring the signal intensity of the methylated and unmethylated probes, the DNA methylation value of each probe was quantified as a b-value ranging from 0 (unmethylated) to 1 (fully methylated). Next, data quality control was implemented for all samples. All 182 samples passed our quality control criteria. Finally, the methylation data were normalized using the functional normalization method to reduce the batch effect problem.

Methylation values

Methylation b-values were converted to M-values for statistical analyses. (M-value = log((B-value)/(1 − (B-value))). M-values were used for consensus clustering and B-values were used for heatmap visualizations, hierarchical clustering, and the generation of a box plot (Figure 1)

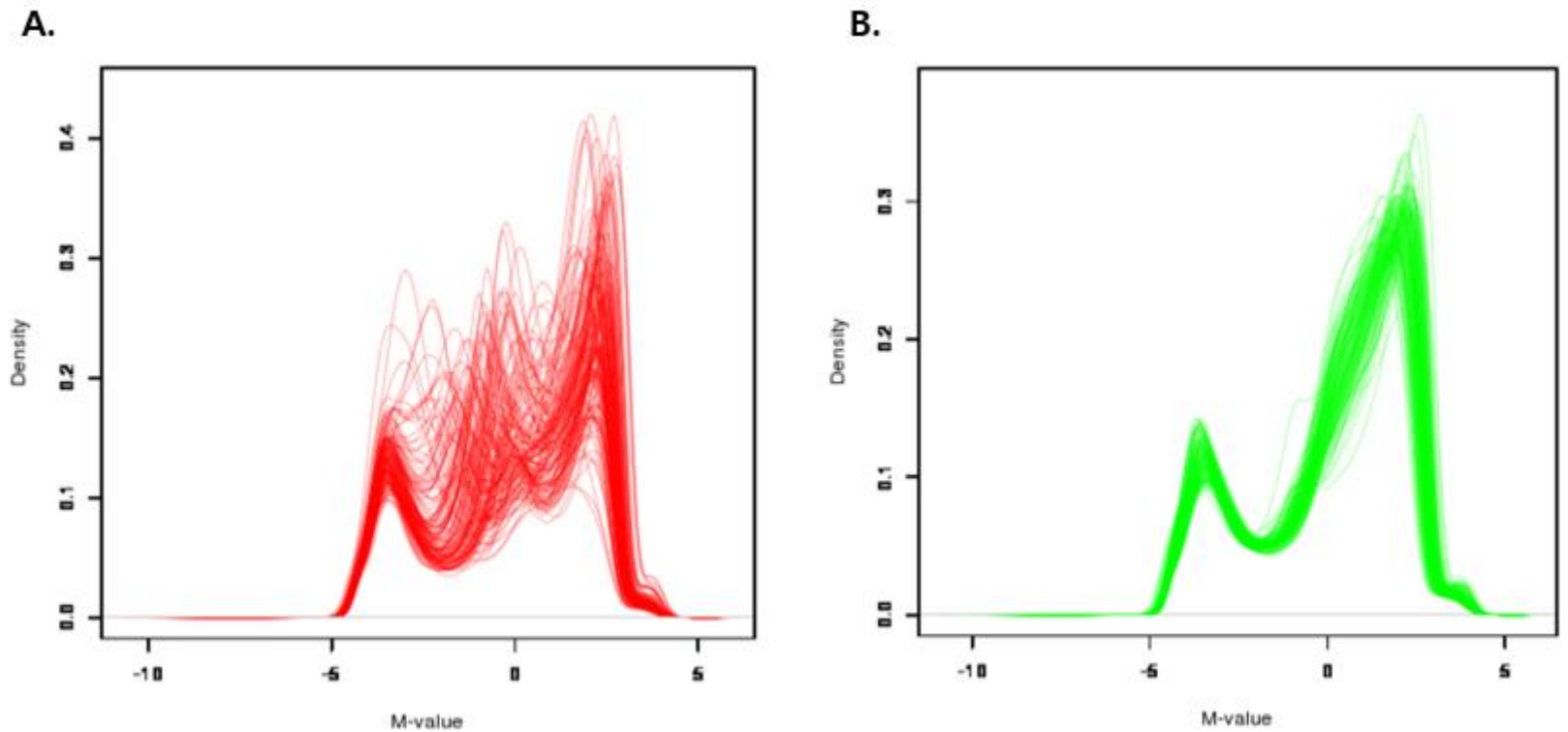


Figure 1. Methylation density plot in 144 HCC samples (A) and 94 normal liver tissue (B).

Consensus clustering

Consensus clustering was performed to identify HCC subgroups using ConcensusClusterPlus (Figure 2A)(42). The top 3,000 most-variable probes (median absolute variation) were used as input and 140 HCC patients were divided into methylation groups 1 and 2 based on their methylation profiles with the top 3000 probes. This algorithm determines "consensus" clusters by measuring the stability of clustering results from the application of a given clustering method to random subsets of the data. In each iteration, 80% of the tumors were sampled, and the k-means algorithm, with the pearson distance metric, was used with k =2 to k =10 groups; these results were compiled over 50 iterations, and the stability of each clustering was determined. All data analysis was performed using the R platform (version ≥ 3.2.1).

Hierarchical clustering and Principal component analysis (PCA) analysis

Hierarchical clustering analysis (HCA) was conducted using Gene cluster 3.0 and the clustering results were visualized using Java TreeView 1.1.6 (Figure 2B). PCA was performed using the 'prcomp' R package in R.

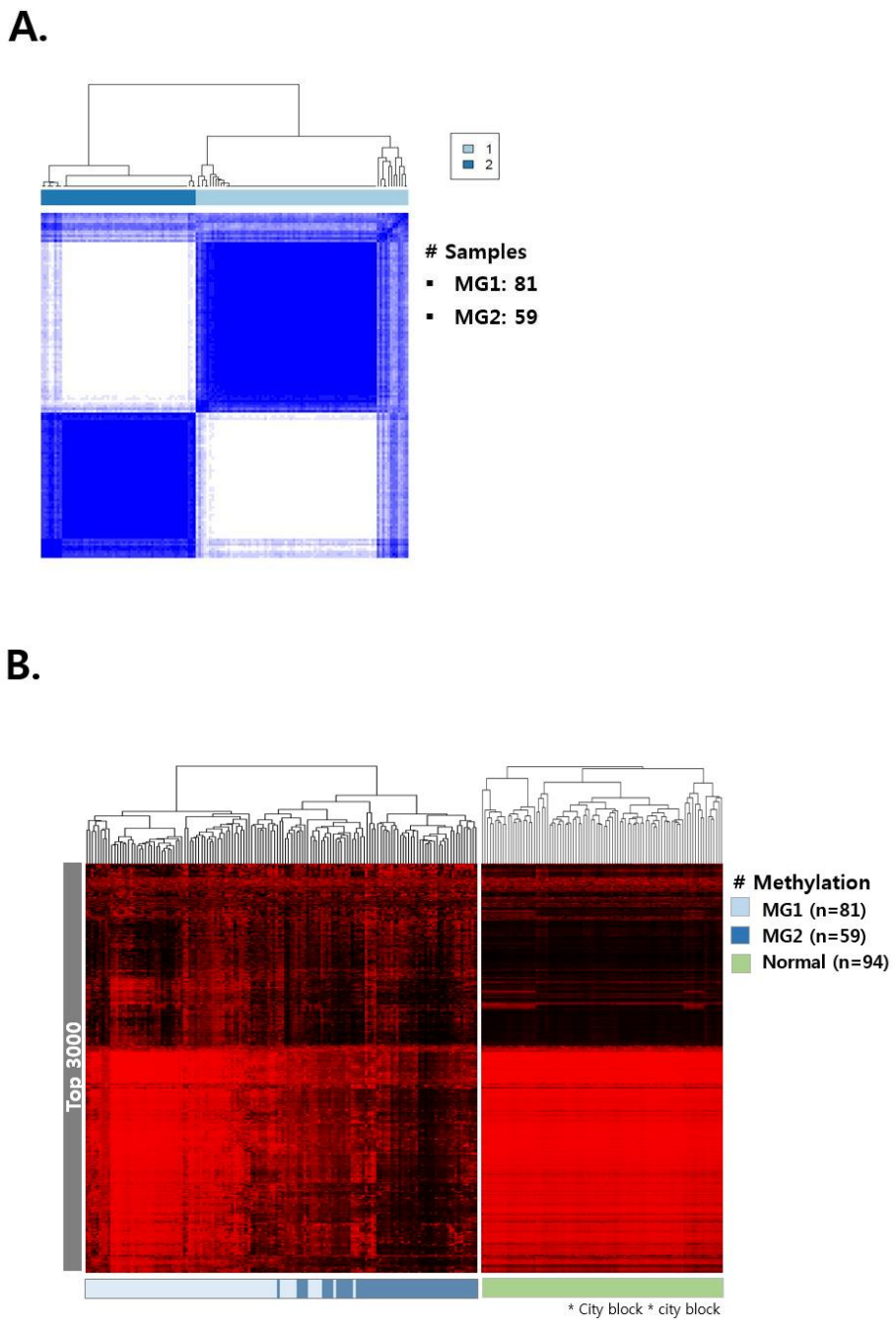


Figure 2. (A) Consensus clustering of hepatocellular carcinoma (HCC) tumors (MAD 3K) (B) Hierarchical clustering of HCC tumors. MG1, methylation group 1; MG2, methylation group 2.

Prognostic marker selection using Random Forest

The Random Forest method is based on a machine learning algorithm that was developed to model nonlinear effects. We used Random Forest R package in R to discover probes that could differentiate HCC groups with good and poor prognosis and performed five-fold cross validation by randomly splitting the HCC tumor samples into four training cohorts and one test cohort. We built the Random Forest model and the following options were used: number of trees = 1000; importance = T (estimating the importance of prediction variables). The prediction rate of each group was estimated by the confusion matrix (predicted versus the observed samples) and the performance of each model was measured based on the area under the curve (AUC), sensitivity, and specificity values (Figure 3).

By considering the Gini index, the total decrease in probe impurity, the accuracy of variable the importance plot, and the dot-chart of variable importance as measured by a Random Forest, we measured the importance value of each probe. Through this, we selected the top 50 probes that separated the two groups, namely of methylation groups 1 and 2 with high performance. Comparing the performance efficiency from one probe to the top 50 probes, the minimum number of probes with the maximum efficiency was selected by considering the AUC value (Figure 4).

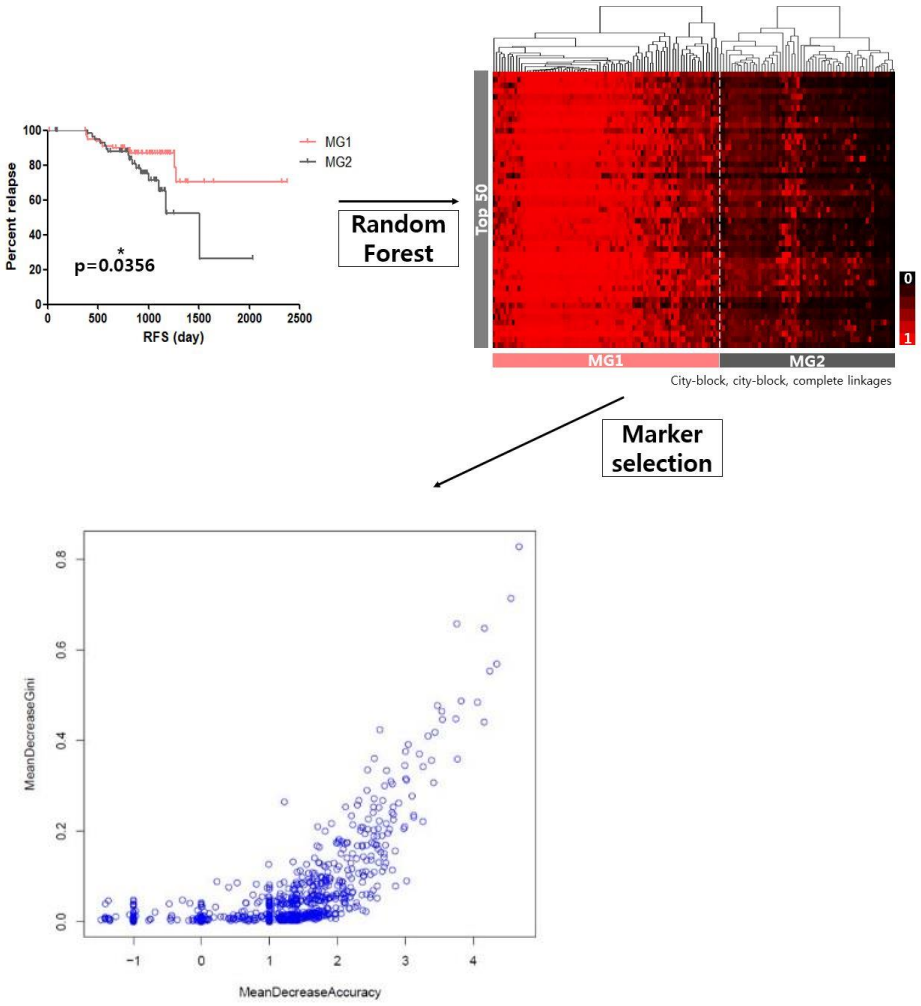
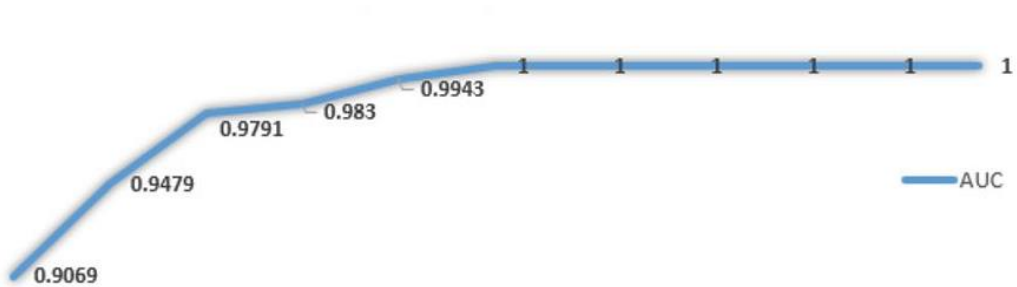


Figure 3. Significant probe selection between the methylation two groups (MAD 3K) of hepatocellular carcinoma.



No. of probes	Top 1	Top 2	Top 3	Top 4	Top 5	Top 6	Top 10	Top 20	Top 30	Top 40	Top 50
Accuracy	0.862	0.893	0.929	0.963	0.963	1	1	1	0.964	1	1
Sensitivity	0.917	0.833	0.917	0.906	1	1	1	1	1	1	1
Specificity	0.824	0.938	0.938	1	0.938	1	1	1	0.938	1	1
AUC	0.907	0.948	0.979	0.983	0.994	1	1	1	1	1	1
95% CI	0.6834 - 0.9611	0.7177 - 0.9773	0.765-0.9912	0.8103 - 0.9991	0.8103 - 0.9991	0.8766 -1	0.8766 -1	0.8766 -1	0.8103 - 0.9991	0.8766 -1	0.8766 -1

Figure 4. Performance evaluation by number of probes. AUC, area under the curve.

Gene ontology and pathway analysis

We searched enriched pathways and performed gene ontology analysis using innateDB (http://www.innatedb.com/). In this database, we used Gene Ontology (GO) biological processes for gene ontology analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome resources for pathway analysis. The significant cut-off criteria was a p-value < 0.05.

Annotation

RefSeq genes and all annotation files of repeat elements, CpG islands, and genomic regions (hg19) were downloaded from the UCSC genome browser. Moreover, 850K EPIC probes were downloaded from the Illumina website. The annotation file of the EPIC array was downloaded from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4864062/(43).

Statistical analysis

The data cut-off date was May 31, 2018. The statistical analysis of categorical variables was performed using the Pearson's χ^2 test or Fisher's exact test. The median durations of relapse-free survival (RFS) were calculated by the Kaplan–Meier method. Comparisons between different groups were performed by log-rank tests. Multivariate analyses were performed using a Cox regression model for relapse-free survival

to identify independent factors and adjust for baseline characteristics. Two-sided *P* values of < 0.05 were considered significant. All analyses were performed using SPSS for Windows, version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Patient selection and baseline characteristics

Of 184 patients, tumor tissue was inadequate to extract DNA for two patients, and therefore, these patients were excluded. Of the remaining 182 patients, we excluded 42 who presented with recurrence within 1 year after surgery (Figure 5). Finally, of the enrolled 140 patients, we classified the samples into two groups according to DNA methylation patterns. The baseline characteristics of the patients (105 males and 35 females) are shown in Table 1. The median age was 57.5 years (range 32–79). HBV (N = 119, 85%) was the most frequent etiology of HCC. The median size and number of tumor nodules were 4.0 cm (range, 1–15) and 1 (range, 1–6), respectively. All patients presented with a Child-Pugh class of A. Until the end of follow-up, tumor recurrence occurred in 28 patients (20.0%). Of these patients, 24 had intrahepatic recurrence, and four had extrahepatic recurrence.

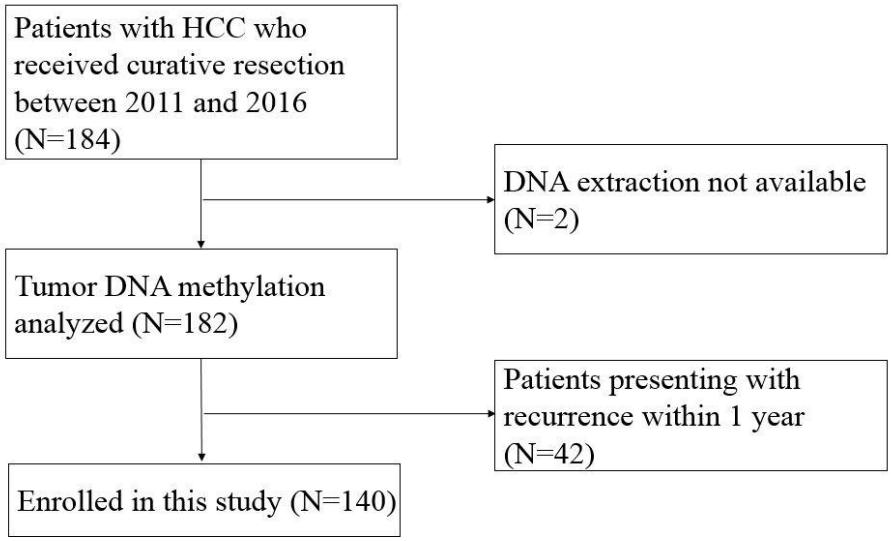


Figure 5. Flow diagram of enrolled hepatocellular carcinoma (HCC) patients.

Table 1. Baseline characteristics of hepatocellular carcinoma patients

	Total (N = 140) (%)
Sex	
Male	105 (75.0)
Female	35 (25.0)
Median age	57.5 (range, 32-79)
Age (y)	
≤ 65	107 (76.4)
> 65	33 (23.6)
HBsAg	
Positive	119 (85.0)
Negative	21 (15.0)
Anti-HCV	
Positive	13 (9.3)
Negative	127 (90.7)
Alcohol abuse	
No	136 (97.1)
Yes	4 (2.9)
Serum albumin (g/dL)	
< 3.3	0(0)
≥ 3.3	140 (100)
Serum bilirubin (μmol/L)	
< 1.2	116 (82.9)
≥ 1.2	24 (17.1)
Serum ALT (IU/L)	
< 50	111 (79.3)
≥ 50	29 (20.7)
Serum AST (IU/L)	
< 50	120 (85.7)
≥ 50	20 (14.3)
Platelet (10^9/L)	
< 100	6 (4.3)
≥ 100	134 (95.7
PT (%)	

< 80	7 (5.0)
≥ 80	133 (95.0)
Child-Pugh grade	
A	0(0)
B	140 (100)
Liver histology	
Normal	7 (5.0)
Chronic hepatitis	103 (73.6)
Cirrhosis	30 (21.4)
AFP (ng/mL)	7 (range, 0–76200)
AFP (ng/mL)	
≤ 100	106 (75.7)
> 100	34 (24.3)
PIVKA-II (mAU/mL)	
PIVKA-II (mAU/mL)	
≤ 40	45 (32.4)
> 40	94 (67.6)
Resection margin	
< 1 cm	90 (64.3)
≥ 1 cm	50 (35.7)
Maximal tumor size (cm)	4.0 (1–15)
Size of largest nodules, cm, no. (%)	
≤ 3	60 (42.9)
3–5	48 (34.3)
≥ 5	32 (22.9)
Number of nodules	
Single	128 (91.4)
Multiple	12 (8.6)
Venous invasion	
Absent	99 (70.7)
Present	41 (29.3)
Tumor encapsulation	
Absent	29 (21.7)
Present	101 (78.3)
Histologic differentiation	
Well-differentiated	64 (45.7)
Moderately-differentiated	58 (41.4)
Poorly-differentiated	18 (12.9)

Milan criteria	
Within	107 (76.4)
Beyond	33 (23.6)
MoRal criteria	
Low	104 (74.8)
High	35 (25.2)

HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PT, prothrombin time; AFP, alpha-fetoprotein; PIVKA-II, prothrombin induced by vitamin K absence or antagonist-II

Characteristics of patients in methylation groups 1 and 2

Table 2 and 3 shows comparisons between methylation group 1 and 2. We first analyzed whether there was a difference based on the tested 13 host factors (sex, age, HBV status, HCV status, alcohol abuse, preoperative serum albumin, bilirubin, ALT, AST, platelet counts, PT, Child-Pugh grade, and nontumorous liver histology; Table 2) and the nine tumor factors (serum AFP level, PIVKA-II level, resection margin, tumor size, multinodularily, venous invasion, tumor encapsulation, histologic differentiation, Milan criteria, and MoRAL criteria; Table 3), which have been linked to recurrence and prognosis, between methylation groups 1 and 2. None of the host factors were significantly different between methylation groups 1 and 2. In contrast, there were differences between these groups in terms of resection margin and number of tumor nodules. The proportion of patients with a tumor resection margin $\geq$ 1 cm was lower in methylation group 2 (44.4 vs 23.7%, methylation group 1 vs 2; p = 0.012). Further, multinodularity was more frequently observed in methylation group 2 (3.7 vs 15.3%, methylation group 1 vs 2; p = 0.028). We calculated the MoRAL score using serum PIVKA-II and AFP levels(44). In methylation group 1, PIVKA-II levels were unknown in one patient. Comparing methylation groups 1 and 2, there were 53 (76.8%) and 34 (69.4) patients with a low MoRAL ($\leq$ 314.8) score (P = 0.367), respectively.

Table 2. Univariate analysis of host factors associated with late recurrence in hepatocellular carcinoma

	Methylation group 1 (N = 81) (%)	Methylation group 2 (N = 59) (%)	P value
Sex			0.277
Male	58 (71.6)	47 (79.7)	
Female	23 (28.4)	12 (20.3)	
Median age	57(range, 32–79)	58 (range, 40–79)	0.876
Age (y)			0.715
≤ 65	61 (75.3)	46 (78.0)	
> 65	20 (24.7)	13 (22.0)	
HBsAg			0.303
Positive	71 (87.7)	48 (81.4)	
Negative	10 (12.3)	11 (18.6)	
Anti-HCV			0.058
Positive	5 (6.2)	8 (13.6)	
Negative	76 (93.8)	51 (86.4)	
Alcohol abuse			0.638
No	68 (97.1)	48 (98.0)	
Yes	2 (2.9)	1 (2.0)	
Serum albumin (g/dL)			

< 3.3	0 (0)	0 (0)	
≥ 3.3	81 (100)	59 (100)	
Serum bilirubin (μmol/L)			0.959
< 1.2	67 (82.7)	49 (83.1)	
≥ 1.2	14 (17.3)	10 (16.9)	
Serum ALT (IU/L)			0.606
< 50	63 (77.8)	48 (81.4)	
≥ 50	18 (22.2)	11 (18.6)	
Serum AST (IU/L)			0.485
< 50	68 (84.0)	52 (88.1)	
≥ 50	13 (16.0)	7 (11.9)	
Platelets (10^9/L)			0.690
< 100	3 (3.7)	3 (5.1)	
≥ 10	78 (96.3)	56 (94.9)	
PT (%)			1.000
< 80	4 (4.9)	3 (5.1)	
≥ 80	77 (95.1)	56 (94.9)	
Child-Pugh grade			
A	0 (0)	0 (0)	
B	81 (100)	59 (100)	

Liver histology			0.087
Normal	2 (2.5)	5 (8.5)	
Chronic hepatitis	65 (80.2)	38 (64.4)	
Cirrhosis	14 (17.3)	16 (27.1)	

HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PT, prothrombin time

Table 3. Univariate analysis of tumor and treatment factors associated with late recurrence in hepatocellular carcinoma

	Methylation group 1 (N = 81) (%)	Methylation group 2 (N = 49) (%)	P value
AFP (ng/mL)	7.5 (range, 0–76200)	5.9 (range, 1–10780)	0.879
AFP (ng/mL)			0.596
≤ 100	60 (74.1)	46 (78.0)	
> 100	21 (25.9)	13 (22.0)	
PIVKA-II (mAU/mL)	67 (range, 16–41439)	180 (range, 17–67885)	0.160
PIVKA-II (mAU/mL)			0.441
≤ 40	28 (35.0)	17 (28.8)	
> 40	52 (65.0)	42 (71.2)	
Resection margin			0.012
< 1cm	45 (55.6)	45 (76.3)	
≥ 1cm	36 (44.4)	14 (23.7)	
Maximal tumor size (cm)	3.0 (range, 1–12)	4.0 (range, 1–15)	0.150
Size of largest nodules, cm, no. (%)			0.905
≤ 3	36 (44.4)	24 (40.7)	
3–5	27 (33.3)	21 (35.6)	
≥ 5	18 (22.2)	14 (23.7)	
Number of nodules			0.028

Single	78 (96.3)	50 (84.7)	
Multiple	3 (3.7)	9 (15.3)	
Venous invasion			0.517
Absent	59 (72.8)	40 (67.8)	
Present	22 (27.2)	19 (32.2)	
Tumor encapsulation			0.211
Absent	20 (23.5)	8 (16.0)	
Present	59 (72.7)	42 (84.0)	
Histologic differentiation			0.653
Well-differentiated	35 (43.2)	29 (49.2)	
Moderately-differentiated	34 (42.0)	24 (40.7)	
Poorly-differentiated	12 (14.8)	6 (10.2)	
Milan criteria			0.659
Within	63 (77.8)	44 (74.6)	
Beyond	18 (22.2)	15 (25.4)	
MoRal criteria			0.214
Low	63 (78.8)	41 (69.5)	
High	17 (21.2)	18 (30.5)	

AFP, alpha-fetoprotein; PIVKA-II, prothrombin induced by vitamin K absence or antagonist-II

Comparisons of methylation profiles between methylation groups 1 and 2

The methylation profile of methylation group 2 was the most distinct from the nontumorous liver tissue. In contrast, patient samples in methylation group 1 had similar methylation profiles as these control liver tissues. These epigenetic changes were associated with signal transduction, mitogen-activated protein kinase (MAPK), and G protein-coupled receptor (GPCR) signaling pathways, as summarized in Table 4. When we interpreted sets of genes based on the gene ontology system of classification, the top 50 probes were found to be related to catabolic process, neurotransmitter secretion, regulation of molecular function, Wnt signaling pathway, regulation of cell migration, mRNA metabolic process, small GTPase mediated signal transduction, regulation of gene expression, and regulation of apoptotic process. Comparing significantly altered hyper- and hypo-methylated CpG sites, we identified hypo-methylated CpG sites located within introns (45%). Moreover, there were 709 (69%) significantly hyper-methylated CpG sites located within promotor regions (Table 5).

Table 4. Pathways associated with DNA methylation in hepatocellular carcinoma tumors

Pathway analysis (n: 3226 Refseq gene)	Source	No. of genes	P-value
Neuronal system	REACTOME	54	2.92E-13
Transmission across chemical synapses	REACTOME	36	1.61E-08
NRAGE signals death through JNK	REACTOME	13	3.56E-06
Signaling by Rho GTPases	REACTOME	22	1.25E-05
Extracellular matrix organization	REACTOME	36	3.71E-05
Axon guidance	REACTOME	42	5.75E-05
Pathways in cancer	KEGG	35	0.004137
Calcium signaling pathway	KEGG	22	0.005238
Signal transduction	REACTOME	157	0.006263
Cell adhesion molecules (CAMs)	KEGG	16	0.030841
MAPK signaling pathway	KEGG	25	0.03735
GPCR downstream signaling	REACTOME	75	0.040999
Hemostasis	REACTOME	44	0.044817

Table 5. Distribution of genomic regions significantly differentially methylated in hepatocellular carcinoma tumors

		Hypo-methylated probes (N = 1556) (%)	Hyper-methylated probes (N = 1032) (%)
Region			
Gene	Exon	289 (19)	331 (32)
	Intron	700 (45)	396 (38)
	Enhancer	35 (2)	26 (3)
CGI	Promoter	47 (3)	709 (69)
	Intragenic	125 (8)	70 (7)
	Intergenic	60 (4)	83 (8)
	nonCGI promoter	99 (6)	31 (3)
	Shore	189 (12)	111 (11)
	Shelf	97 (6)	7 (1)
Repetitive element	DNA transposon	29 (2)	4 (0.4)
	LINEs	82 (5)	2 (0.2)
	SINEs	48 (3)	1 (0.1)
	LTRs	70 (4)	4 (0.4)
	Simple repeat	10 (1)	10 (1)

CGI, CpG islands; LINEs, Long interspersed nuclear elements; SINEs, Short interspersed nuclear elements; LTR, Long terminal repeats

Effect of DNA methylation on RFS

At the time of analysis, 28 (23.5%) patients had experience disease recurrence. Among them, 24 (85.7%) developed intrahepatic recurrence and four (14.3%) developed extrahepatic recurrence. The median follow-up period was 971 days (range, 374–2457). In methylation groups 1 and 2, recurrence was observed in 12 (14.8%) and 16 (27.1%) patients, respectively. Figure 6 shows the cumulative Kaplan–Meier curve analysis of RFS among the 140 patients in this study. Overall, the median RFS was not reached. However, comparing groups, the median RFS in methylation group 1 was not reached, whereas the median RFS in group 2 was 1505 days (95% CI: 1111–1898). There was also a statistically significant difference in the median RFS between the two methylation groups (not reached vs 1505 days, p = 0.036; Figure 7). We next evaluated the risk factors that affect RFS. The median RFS of patients with a single nodule compared to those with multiple nodules was significantly longer (not reached vs 1270 days, respectively, p = 0.015; Figure 8). In addition, patients with preoperative thrombocytopenia (platelet $< 100 \times 10^9$/L) had a worse RFS compared to patients without thrombocytopenia (921 days vs not reached, respectively, p = 0.045; Figure 9). As shown in Table 6, methylation group 2, thrombocytopenia, and multinodularity were associated with poorer RFS. Considering only methylation group 1, the RFS for patients with a single tumor and platelet $\geq 100 \times 10^9$/L was longer than that for patients with single tumor and

preoperative thrombocytopenia and for patients with multinodular tumors and platelet $\geq 100 \times 10^9$/L (not reached, 545 days, and 1270 days, respectively; p = 0.006). However, there was no difference in RFS between patients within and beyond the Milan criteria. Tumor markers including AFP and PIVKA-II were also not helpful to predict tumor recurrence. Further, the MoRAL score was not predictive of RFS based on univariate analysis. By multivariate analysis, we excluded the number of nodules, which correlated with methylation group. Finally, between methylation groups and thrombocytopenia, only methylation group was a significant predictor of recurrence based on multivariate analysis (p = 0.039, HR 2.219, 95% CI: 1.036–4.754) (Table 7).

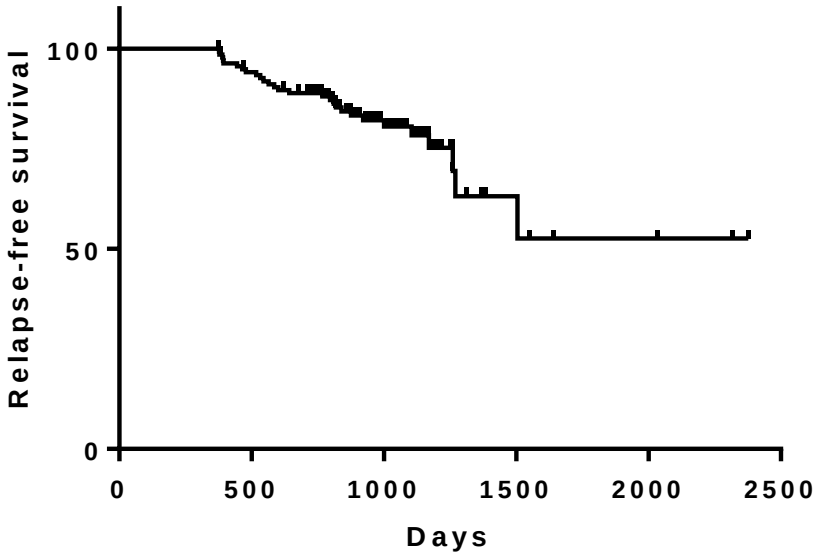


Figure 6. Cumulative relapse free survival curve of all 140 hepatocellular carcinoma patients in the cohort based on Kaplan–Meier analysis.

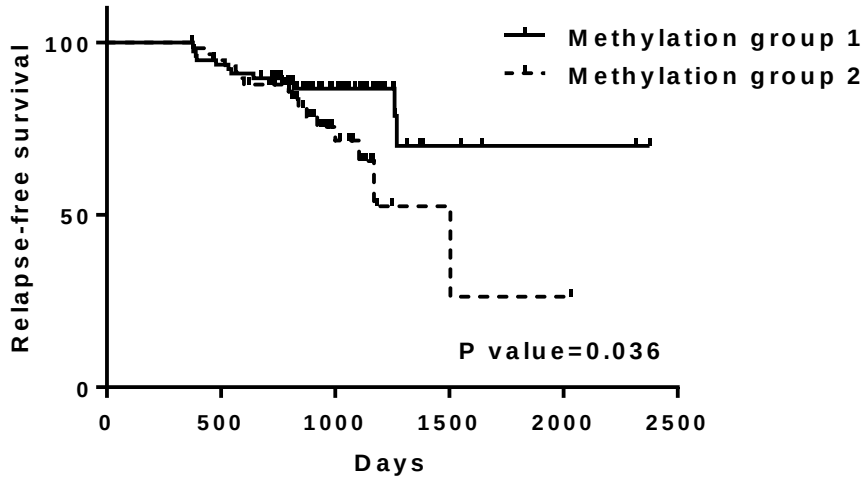


Figure 7. Cumulative relapse free survival curve of hepatocellular carcinoma patients according to methylation group based on Kaplan–Meier analysis.

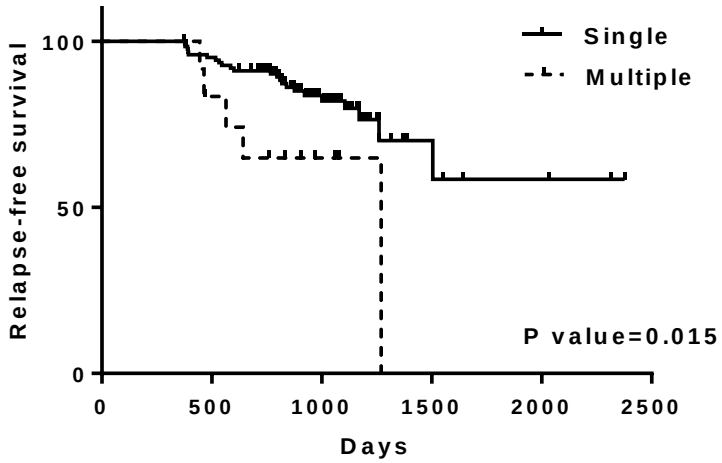


Figure 8. Cumulative relapse free survival curve of hepatocellular carcinoma patients according to number of nodules based on Kaplan–Meier analysis.

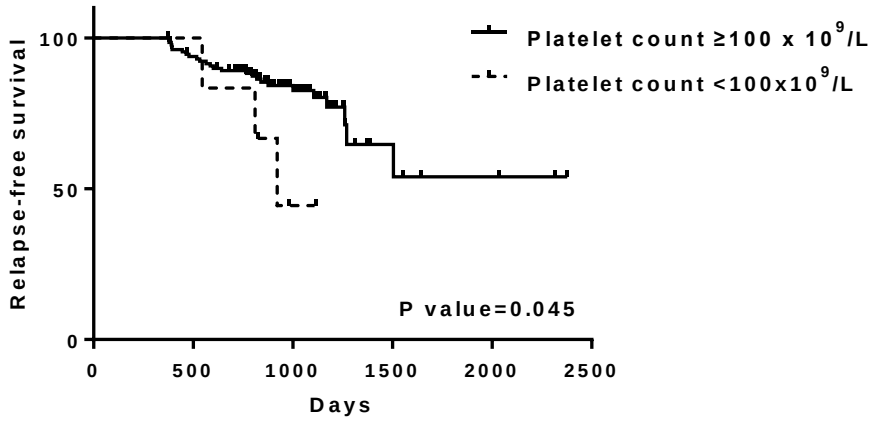


Figure 9. Cumulative relapse free survival curve of hepatocellular carcinoma patients according to platelet count based on Kaplan–Meier analysis.

Table 6. Analysis of prognostic factors associated with relapse free survival in hepatocellular carcinoma patients

	Median RFS Days (95% CI)	Univariate p-value
Methylation group		0.036
1	not reached	
2	1505 (1111–1898)	
Sex		0.065
Male	1505	
Female	not reached	
Age (y)		0.316
≤ 65	not reached	
> 65	1270 (1249–1290)	
HBsAg		0.162
Positive	not reached	
Negative	1170 (753–1586)	
Anti-HCV		0.124
Positive	not reached	
Negative	not reached	
Alcohol abuse		0.416
No	not reached	
Yes	not reached	
Serum bilirubin (μmol/L)		0.930
< 1.2	not reached	
≥ 1.2	not reached	
Serum ALT (IU/L)		0.129
< 50	not reached	
≥ 50	1505 (508–2501)	
Serum AST (IU/L)		0.161
< 50	not reached	
≥ 50	not reached	
Platelet (10^9/L)		0.045
< 100	921 (703–1138)	
≥ 100	not reached	
PT (%)		0.468
< 80	not reached	

≥ 80	not reached	
AFP (ng/mL)		0.437
≤ 100	not reached	
> 100	1270	
PIVKA-II (mAU/mL)		0.847
≤ 40	not reached	
> 40	not reached	
Liver histology		0.756
Normal	1170 (741–1598)	
Chronic hepatitis	not reached	
Cirrhosis	1260	
Resection margin		0.141
< 1 cm	1505	
≥ 1 cm	not reached	
Number of nodules		0.015
Single	not reached	
Multiple	1270	
Venous invasion		0.148
Absent	not reached	
Present	1270 (988–1551)	
Tumor encapsulation		0.316
Absent	1260	
Present	not reached	
Histologic differentiation		0.727
Well-differentiated	not reached	
Moderately-differentiated	not reached	
Poorly-differentiated	1505	
Milan criteria		0.355
Within	not reached	
Beyond	1505	
MoRal criteria		0.518
Low	not reached	
High	1505	

RFS, recurrence-free survival; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PT, prothrombin time; AFP, alpha-fetoprotein; PIVKA-II, prothrombin induced by vitamin K absence or antagonist-II

Table 7. Multivariate analysis of prognostic factors associated with relapse-free survival in hepatocellular carcinoma patients

	Median RFS Days (95% CI)	Univariate P value	Multivariate hazard ratio (95% CI) P value
Methylation group		0.036	0.039
1	Not reached		Ref
2	1505 (1111-1898)		2.219 (1.036-4.754)
Platelet (10^9/L)		0.045	0.091
<100	921 (703-1138)		Ref
≥100	Not reached		0.350 (0.104-1.183)
Number of nodules		0.015	
Single	Not reached		
Multiple	1270		
Liver cirrhosis		0.708	
Absent	Not reached		
Present	1260		

Discussion

The major finding of the present study was that late recurrence in patients who have received curative resection for HCC can be predicted by DNA methylation analysis. Specifically, in our study, the median RFS of methylation group 1 was longer than that of methylation group 2.

A major reason for poor prognosis with HCC is the high probability of recurrence after surgical resection. We excluded patients who had presented with recurrence early (< 1 year after resection) because different risk factors are considered involved in early and late recurrence. Previous studies showed that early recurrence most likely originates from the subclinical metastasis of primary tumors, whereas late recurrence might represent *de novo* primary HCC or multicentric disease. The presence of microsatellite and microvascular invasion, increasing tumor stage, and elevated AFP levels is also known to be related to early recurrence, whereas cirrhosis, multinodularity, and hepatitis activity are risk factors for late recurrence (32, 33). In our study, multinodularity was not only related to worse RFS, but also associated with methylation group 2. Thus, the DNA methylation signature might reflect key background tumor features. Based on methylation profiles in our study, more patients in methylation group 2 than in methylation group 1 had more multi-nodular tumors, which might suggest that changes in DNA

methylation play a critical role in driving aggressive feature such as multinodularity.

In our study, thrombocytopenia was also predictive of late recurrence based on univariate analysis. Most patients with HCC have cirrhosis, which is primarily caused by chronic liver inflammation. Liver cirrhosis finally leads to portal hypertension and hypersplenism and causes thrombocytopenia. Furthermore, platelet counts and many noninvasive indices that are considered critical disease components are well-known diagnostic indicators to predict liver fibrosis and cirrhosis (45-48). Some studies have shown that platelet levels comprise a predictor of liver-related death and HCC occurrence (49-51). Previous studies also demonstrated that a low platelet level results in poorer prognosis in HCC patients after liver resection (52, 53). In our study, thrombocytopenia was a predictive marker of late recurrence, although this was not statistically significant based on multivariate analysis.

The strength of this study was the large number of HCC cases recruited, and that most cases were HBV-related. To develop the methylation signature, we profiled 140 HCCs using high-density methylation arrays. There have been previous studies that have studied epigenetic prognostic markers in HCC. Song et al showed substantial changes in DNA methylation at the genome-wide level in HCC, but the sample size was small including 27 HCC and 20 adjacent normal liver tissues(54). In addition, Shen et al identified aberrant DNA methylation

profiles across the genome of HCC tissue that were predominantly related to HCV infection(55). Compared to previous studies looking at epigenetic prognostic markers in HCC, our study population was larger and consisted of predominantly HBV-infected patients. Of the enrolled patients, the most common etiology of HCC was HBV (N = 119, 85.0%). Because chronic HBV infection is still the major cause of this disease in all Asia-Pacific countries except for Japan, our results could be especially applied to HBV-infected HCC patients in Asia. Chronic HBV infection can lead to chronic liver inflammation and the accumulation of genetic alterations that result in the oncogenic transformation of hepatocytes. HBV can also sensitize hepatocytes to oncogenic transformation by causing genetic and epigenetic changes in host chromosomes. HBV DNA can insert into host chromosomes, and recent large-scale whole-genome sequencing studies reveled recurrent HBV DNA integration sites that might play important roles in the initiation of hepatocellular carcinogenesis. HBV can also cause epigenetic changes by altering the methylation status of cellular DNA, the post-translational modification of histones, and the expression of microRNAs. These changes can also cause eventual hepatocellular transformation.

An additional strength of this study was that the characteristics of the enrolled patients were relatively homogenous at early clinical stages. For all patients, the Child-Pugh class was A. Moreover, patients had predominantly uninodular disease (N = 128, 91.4%) and a median tumor

size of 4.0 cm, representing the majority of cases at early clinical stages. Nontumorous liver histology mainly composed of normal liver (N = 7, 5.0%) and chronic hepatitis (N = 103, 73.6%). Based on this, it was difficult to predict recurrence because many patients did not exhibit well-known risk factors for relapse in this population. However, our methylation profiles provided more information for patients at a higher risk of late recurrence.

Our data is consistent with the multistep process of HCC, which results in the accumulation events as disease progresses (56, 57). Although all 3000 methylation probes of group 2 patients with worse RFS were found to be different from the methylation profiles of nontumorous liver tissues, patients in methylation group 1 had a similar methylation profile to that of the nontumorous liver tissues, leading to a better disease-free survival profile. Hence, the methylation profile of HCC could supplement existing strategies incorporating molecular characteristics to categorize groups of patients with different prognoses. These epigenetic changes were associated with signal transduction, mitogen-activated protein kinase (MAPK), and G protein-coupled receptor (GPCR) signaling pathways. Furthermore, there were 709 (69%) significantly hypermethylated CpG sites located within promotor regions. Promoter hypermethylation is an important mechanism associated with the repression of gene transcription in cancer. In addition, we investigated the differences in RFS between methylation groups 1 and 2.

At first, a metabolism pathway including the pentose pathway in methylation group 2 was more active, although the pentose pathway in methylation group 1 was suppressed compared to that in methylation group 2 and even in non-tumorous liver tissue (data not shown). Second, genes involved in cell cycle check points can be dysregulated by aberrant DNA methylation. These changes could result in the poor prognosis of methylation group 2. Further studies are thus warranted to reveal which of these genes and pathways regulate aberrant methylation. In the future, we plan to compare methylation profiles with respect to primary and recurrent tumors.

To simplify procedures, we selected the top six probes to classify methylation groups. These were associated with the same prognostic performance as the 3000 probes used for methylation profiles. However, for clinical use, we need to prove that the top six probes are predictive markers based on a validation cohort. Of these, there are two coding regions representing the gene IDs of *MAGEL2* and *MYT1L*, which should be investigated as potential drug targets. Of the four noncoding lesions in the top six probes, we could hypothesize that DNA methylation in noncoding lesions is induced through direct or indirect changes in chromatin structure, which play crucial roles in gene regulation.

The epigenetic changes observed in our study indicate that HCC exhibits specific methylation signatures with potential clinical application for HCC diagnosis and prognosis. After resection in HCC,

there is no standard adjuvant treatment. Although clinical trials for early stage HCC after resection have been conducted, no adjuvant therapy has shown benefits for survival. Our data could be used to help identify appropriate candidates for adjuvant therapy. To provide more personalized therapy for patients at a higher risk of late recurrence and to avoid unnecessary overtreatment, methylation profiles could thus provide valuable information for clinicians. After resection, we can consider preemptive liver transplantation before recurrence in methylation group 2, because the recurrence risk is higher for patients in methylation group 2 compared to that for group 1.

Conclusions

In conclusion, there was a significant association between methylation profiles and RFS in HCC patients after hepatic resection. The methylation profile of methylation group 2 with worse prognosis was distinctively different from the nontumorous liver tissue. Further studies will be required to validate the prognostic performance of our methylation-based model using an independent data set.

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요약(국문초록)

서론: 간세포암은 전세계적으로 가장 흔하게 발생하는 암종 중의 하나이며, 이러한 간세포암의 발병 기전에는 후성유전체의 변화가 중요한 역할을 하는 것으로 알려져 있다. 본 연구에서는 간세포암 유전체 메틸화 변이가 수술 후 1년 이후에 일어나는 재발과의 관련성에 대해서 알아보고자 하며, 이러한 유전체 메틸화가 재발을 예측하는 인자로서 역할을 할 수 있는지 알아보고자 한다.

방법: 2011년부터 2016년까지 서울대학교병원에서 근치적 목적으로 수술을 시행받은 총 184명의 간세포암 환자를 전향적으로 등록하여 검체를 수집하였다. Illumina Infinium HumanMethylation EPIC 850K BeadChip (Illumina, CA, USA)으로 간암 조직과 인근 정상 간 조직에서 유전자 메틸화를 분석하였다.

결과: 총 184명의 환자 중에서, 조직에서 유전자 추출에 실패한 2명의 환자 및 수술 후 1년 이전에 재발한 42명의 환자는 분석에서 제외되었다. 나머지 140명의 환자에서 consensus clustering 기법을 이용하여 메틸화 변화를 바탕으로 크게 두 그룹으로 나누었다. 메틸테이션 그룹 1은 81명(57.8%), 그룹 2는 59명(42.2%)이었고, 두 그룹간 메틸화의 차이는 명백하였다. 그룹 1의 유전자 메틸화는 정상 간 조직과 유사한 경향을 보였으나, 그룹 2의 메틸화는 정상 간조직과 극명한 차이가 있었으며, 정상 간조직에 비교하여

메틸레이션이 감소된 경향을 보였다. 논문의 결과 분석 시점에서는 총 28명(23.5%)의 환자가 재발한 상태였다. 그룹 1에서는 12명(14.8%), 2에서는 16명(27.1%)이 재발을 경험하였다. 무병생존기간에 대한 분석에서는 그룹 1의 무병생존기간은 아직 중앙값에 도달하지 않았으며, 그룹 2의 무병생존기간 중앙값은 1505일로 보고되어 그룹 1이 통계적으로 유의하게 더 긴 무병생존기간을 보였다(유의확률 0.036). 그 외에, 수술 전 혈소판 감소(10만/L 미만)가 있는 환자들의 무병생존기간 중앙값이 921일로, 혈소판 감소가 없는 환자들에 비해서 짧았다(유의확률 0.045). 그러나, 무병생존기간에 대한 다변량 분석에서는 유전자 메틸화 차이만이 수술 1년 이후 재발을 예측하는 유의한 인자였다.

결론: 본 연구에서는 간세포암의 유전체 메틸화 변화가 수술 1년 이후의 재발과 관련성이 있음을 확인하였다. 메틸테이션 그룹 2는 그룹 1에 비교해서 짧은 무병생존기간은 보였다. 본 연구 결과를 바탕으로 1년 이후 재발위험이 높을 것으로 예상되는 환자들을 선정하여, 위험도에 따른 맞춤치료를 하는데 근거를 제시할 수 있을 것으로 기대된다.

주요어: 간세포암; 유전체 메틸화; 후성유전체학; 재발; 예측 인자; 예후

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